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DNA-Dependent Protein Phosphorylation Activity in *Xenopus* Is Coupled to a Ku-Like Protein

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Abstract. DNA-dependent protein kinase (DNA-PK) is a nuclear enzyme and functions as a serine/threonine kinase that has been well characterized in both the human and the mouse. The regulatory subunit of DNA-PK is the Ku autoantigen. To demonstrate that a Ku-like protein is present in *Xenopus* oocytes, we used immunoprecipitation analysis with a monoclonal antibody raised against human Ku antigen and autoimmune serum containing anti-Ku antibodies. Metabolic labeling studies indicate that the Ku-like protein is synthesized mainly in late vitellogenic oocytes. By using a specific peptide substrate for DNA-PK, we demonstrate the activity of a DNA-dependent protein kinase in oocyte extracts. The kinase activity requires the Ku-like protein, since extracts depleted of Ku protein by immunoadsorption with human anti-Ku antibodies fail to demonstrate the DNA-dependent phosphorylation activity. The increased enzyme activity in vitellogenic oocytes may be correlated to the increased levels of Ku protein observed in these oocytes compared to the pre- and early vitellogenic oocytes.

Introduction

DNA-dependent protein kinase (DNA-PK) is a multisubunit enzyme that includes the Ku protein, which is a heterodimer composed of 70-kDa and 80-kDa polypeptide subunits (Dvir *et al.*, 1992; Gottlieb and Jackson, 1993) and a catalytic subunit of ~460 kDa (Blunt *et al.*, 1995). The Ku heterodimer functions as the regulatory component of DNA-PK and binds to the ends of non-specific double-stranded DNA (dsDNA) (Gottlieb and Jackson, 1993). Although DNA-PK has been shown to affect multiple processes, including transcription (Feldmann and Winnacker, 1993; Cao *et al.*, 1994) and DNA repair and recombination (Anderson and Lees-Miller, 1992; Mizuta *et al.*, 1994; Taccioli *et al.*, 1994; Finnie *et al.*, 1995; and Peterson *et al.*, 1995), the *in vivo* targets of this enzyme have not been defined. Recent reports indicate that mice deficient in the 80-kDa subunit of Ku exhibit severe combined immunodeficiency and defective processing of V(D)J recombination intermediates (Nussenzweig *et al.*, 1996; Zhu *et al.*, 1996). These mice are also smaller than their normal littermates (Nussenzweig *et al.*, 1996).

DNA-PK activity has been detected in rabbit reticulocyte lysate; in eggs and oocyte extracts obtained from *Xenopus*, clam (*Spisula*), sea urchins (*Arbacia*); and in cellular extracts of mouse, hamster, and *Drosophila* (see review by Anderson and Lees-Miller, 1992 and reports by Walker *et al.*, 1985; Finnie *et al.*, 1995; and Kanungo *et al.*, 1996a). Much recent work has been done in *Xenopus*. Although the catalytic and regulatory subunits of DNA-PK remain to be characterized in this organism, DNA-dependent phosphorylation of histone during nucleosome assembly has been demonstrated in the oocytes (Kleinschimdt and Steinbeisser, 1991). DNA-PK has been reported to suppress RNA polymerase I transcription in extracts of embryonic kidney cells of *Xenopus* (Kuhn *et al.*, 1995; Labhart, 1995); and the N-terminal domain of *Xenopus* TATA box-binding protein has been shown to be a target of DNA-PK *in vivo* (Labhart, 1996). Furthermore, experiments with extracts of *Xenopus* eggs have indicated that DNA-PK may be involved in the phosphorylation of P1 protein (Someya *et al.*, 1995). We have carried out studies to determine whether the DNA-PK

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activity detected in *Xenopus* is associated with a Ku-like protein, and to evaluate preliminarily whether the enzyme activity varies in different stages of oocytes.

Materials and Methods

Unless indicated, all chemicals were purchased from Sigma. Female African clawed frogs (*Xenopus laevis*) were purchased from Nasco (Wisconsin), and the oocytes were staged according to Dumont (1972). Isolated oocytes were labeled with ^{35}S-methionine, 1 μCi/μl (Amersham) in Barth's modified saline (Gurdon, 1968). Defolliculated full-grown oocytes were homogenized in a Dounce homogenizer (50% v/v) in 50 m*M* HEPES, pH 7.4; 10 m*M* EGTA, 40 m*M* NaCl, 100 m*M* potassium acetate, 8.56 m*M* $CaCl_2$, 2.29 m*M* $MgCl_2$, 277 m*M* glycerol. Centrifugation of the homogenate (12,000 $\times$ *g* $\times$ 30 min, 4°C) yielded a supernatant that was recentrifuged to separate small particulate components from soluble components (35,000 $\times$ *g* $\times$ 60 min, Beckman SW 50.1).

Immunoprecipitation and protein analysis

Oocytes were homogenized in immunoprecipitation buffer (50 m*M* Tris-Cl, pH 7.5; 0.5 *M* NaCl, 0.05% NP 40) containing 1 m*M* phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin and 2.5 μg/ml pepstatin. The homogenate was centrifuged (12,000 $\times$ *g* $\times$ 30 min) and the supernatant added to 2 mg of Protein A Sepharose CL-4B (Pharmacia) coupled to the appropriate antibody. The immunoprecipitates were boiled with protein sample buffer and resolved with SDS-PAGE, 7.5% (Laemmli, 1970) with subsequent autoradiography.

DNA-dependent protein kinase assay

A peptide comprising amino acids 11–24 of human p53 (*EPPLSQEAFADLWKK*) (Anderson, 1993) was used as the specific substrate for the DNA-dependent protein phosphorylation assay. The assay was performed at 20°C in a 15-μl reaction volume containing 10 μl of oocyte extract (140 μg total protein), 75 ng of the desired nucleic acids, 200 μM of peptide substrate, 2 m*M* $MgCl_2$, 130 μM ATP, 1 m*M* dithiothreitol, and 10 μCi of γ-^{32}P ATP (6000 Ci/mmol) (NEN, Du Pont). After 30 min, acetic acid was added to a concentration of 30%, and the reaction product was spotted onto P81 phosphocellulose discs (Whatman). The discs were washed in sequence with 30% acetic acid, 15% acetic acid, and acetone (5 min), and subsequently air dried and counted in a scintillation counter. Reactions without DNA and without substrate or with a nonspecific substrate were always included in the experiments. Subtraction of these values from reactions with added DNA and with added substrate gives the final activity. A time-course experiment showed a linear incorporation of radiolabeled phosphate up to 30 min.

Immunodepletion of Ku from oocyte extracts

Oocyte extracts were depleted of Ku protein by immunoadsorption using Protein A Sepharose beads (Pharmacia) coupled with autoimmune sera (5 μl) containing anti-Ku antibodies and a monoclonal antibody raised against human Ku protein (mAb 162). Thirty microgram IgG (mAb 162) was used for each immunodepletion assay. The monoclonal antibody mAb 162 recognizes a conformational epitope on p80/p70 heterodimer. Therefore, it is effective for immunoprecipitation of native protein (the heterodimer) but is unable to recognize the denatured protein (Wang *et al.*, 1993). Mock-depleted extracts were prepared by treating the extracts with beads coupled to normal human serum. Depletion of other antigens, like Ro and Sm, was performed using the human autoimmune sera previously characterized.

Results

An autoimmune serum containing anti-Ku antibodies and monoclonal antibody 162 immunoprecipitated Ku-like polypeptides from radiolabeled oocytes (Fig. 1a, lane C, and Fig. 1b, lane B′). The polypeptides had electrophoretic mobilities closely approximating those of the Ku protein subunits identified in HeLa cells (Fig. 1a, lane A). Autoimmune sera containing antibodies to Ro and Sm but not to Ku were unable to immunoprecipitate a similar protein from the oocyte extracts (Fig. 1a, lanes D–E). Several other autoimmune sera containing anti-Ku antibodies were also examined. These sera immunoprecipitated Ku-like polypeptides (Fig. 1b) and in some cases additional proteins (Fig. 1b, lane D′). The monoclonal antibody 162 recognizes only the conformational epitope of the Ku heterodimer. Evidently, the conformational epitope for the Ku protein is conserved throughout evolution, because the *Xenopus* Ku protein was recognized by mAb 162, an antibody that detects the native form of Ku protein in mammalian cells. Unfortunately, mAb 162 does not recognize denatured Ku. Therefore an immunoblot analysis could not be performed with this antibody.

In *Xenopus*, the early vitellogenic phase represented by Y1–Y3 oocytes is followed by a vitellogenic phase represented by Y4–Y5 oocytes. When the oocytes are fully grown and no more yolk is deposited, the phase is designated as the post-vitellogenic stage (represented by Y6 oocytes). Immunoprecipitation assays on extracts prepared from radiolabeled oocytes at different stages of growth indicated that the biosynthesis of the Ku-like protein varied during oogenesis (Fig. 1c). The appearance of new protein was not detectable in early vitellogenic (Y1–Y3) oocytes (lanes A–C) but was detected in later stages

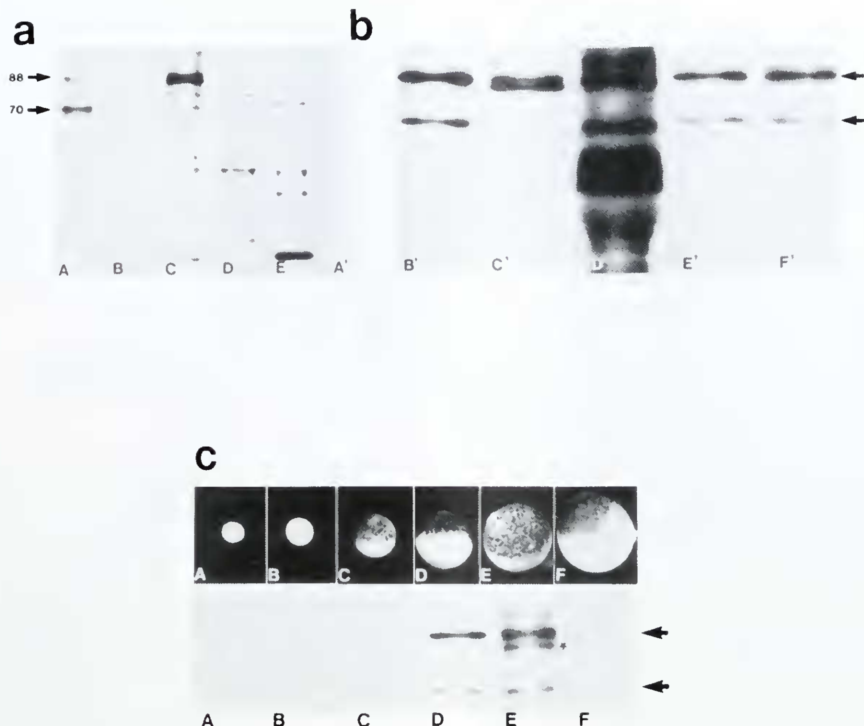

Figure 1. Autoradiogram of immunoprecipitates from oocyte extracts shows Ku-like polypeptides. Stage 5 oocytes were radiolabeled with [^{35}S]-methionine (see Materials and Methods) by incubating the oocytes for 18 h at 18°C. Ten oocytes were used for each immunoprecipitation assay. (a) Ku (p80/p70) immunoprecipitated with a monoclonal antibody (mAb 162) from HeLa cell extract (A); oocyte extracts immunoprecipitated with normal human serum (B), human autoimmune serum (KT) having anti-Ku antibody (C), human autoimmune serum having anti-Ro antibody (D), and human autoimmune serum having anti-Sm antibody (E). (b) Radioimmunoprecipitates of oocyte extracts with pre-immune IgG (A′), mAb 162 (B′), and with human autoimmune sera having anti-Ku antibodies; serum OM (C′), serum HT (D′), serum KT (E′), serum KY (F′). (c) Autoradiogram of immunoprecipitates from oocyte extracts shows Ku-like polypeptides during oogenesis. The OM serum, containing the anti-Ku antibody, was used for the immunoprecipitation assay. The number of oocytes for each stage was standardized so as to use equal amounts of total protein for each immunoprecipitation: Stage I (Y1) (Lane A), stage II (Y2) (Lane B), stage III (Y3) (Lane C), stage IV (Y4) (Lane D), stage V (Y5) (Lane E), and stage VI (Y6) (Lane F). The Ku polypeptides (p80 and p70) are indicated (arrows). Another protein band running slightly faster than p80 (*) is not identified, but may be a degradation product of p80. The unidentified band is not immunoprecipitated by mAb 162 or other autoimmune sera that recognize Ku (Fig. 1b).

(lanes D–E). We have not determined if the increased synthesis observed in the later stages of oogenesis correlates with a stage-specific function or if the oocytes stockpile DNA-PK for use after fertilization. The immunoprecipitated Ku is not as abundant in Y6 oocytes as in Y5 oocytes. This difference may be due to the decreased synthesis of Ku in the full-grown oocyte, Y6. The previously synthesized Ku apparently does not incorporate ^{35}S-methionine, and therefore the intensity of the Ku polypeptides in the radioimmunoprecipitation assay is less than that in Y4 and Y5 oocytes. Because the antibody does not recognize the Ku protein in immunoblots of crude extracts of oocytes, we could not directly quantify the level of Ku protein stored in the oocytes.

Using a specific peptide substrate (derived from the tumor suppressor protein p53), we detected a DNA-dependent phosphorylation activity in oocyte extracts prepared from oocytes at stages Y4–Y6. The peptide was phosphorylated in the presence of sonicated calf thymus DNA, but not in the presence of single-stranded (ss) DNA, total RNA from calf thymus or a 23-mer ss-oligonucleotide (Fig. 2a). This result suggests that double-stranded DNA (dsDNA) activates a kinase that is present in the oocytes. When oocyte extracts were pretreated by immunoprecipitation with anti-Ku antibodies, specific phosphorylation was reduced significantly. Patient sera containing anti-Ku antibodies removed most of the catalytic activity from the oocyte extract, whereas sera containing anti-Sm and anti-Ro antibodies had no significant effect (Fig. 2b). Thus, as with the activity observed in mammalian cells, the DNA-dependent protein kinase activity detected in the oocyte extracts appears to be regulated by a Ku-like protein.

In early vitellogenic oocytes (Y1–Y3), the Ku-like protein is barely detectable as determined by radioimmunoprecipitation. To assay stage-specific DNA-PK activity, we isolated the oocytes at different stages. Extracts prepared from early-stage oocytes had very little DNA-PK activity compared to the vitellogenic (Y4–Y5) and late-vitellogenic (Y6) oocytes (Fig. 3). This correlation between levels of Ku-like protein and DNA-PK activity suggests a functional link.

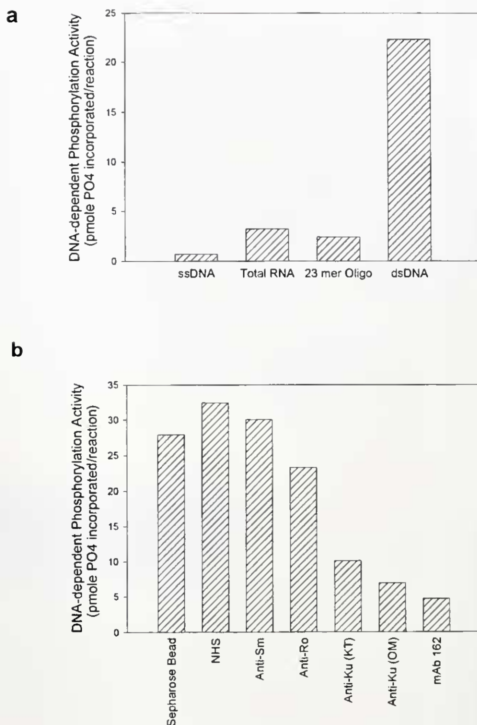

Figure 2. (a) Induction, using nucleic acids, of DNA-dependent protein kinase activity in oocyte extracts to evaluate the nucleotide-dependent enzyme activity. Values obtained from assays of extracts in the absence of any added nucleotide were subtracted from the values obtained from assays in the presence of different nucleotides. Similarly, specific peptide phosphorylation assays were performed by comparing assays with and without the addition of the peptide substrate. Enzyme activity in the crude extract is expressed as picomole phosphate incorporation per reaction containing 200 μM specific peptide substrate; ssDNA (sonicated single-stranded calf thymus DNA), total RNA from calf thymus, 23 mer oligo (single-stranded oligonucleotide) and dsDNA (sonicated double-stranded calf thymus DNA); (b) Ku antigen-depleted oocyte extract shows a significant decrease in DNA-dependent protein kinase activity. The nucleic acid used to induce the kinase activity is sonicated calf thymus dsDNA. Human autoimmune sera with antibodies against Ku, a monoclonal antibody against human Ku (mAb 162), Sm and Ro were used to deplete the respective antigens from oocyte extracts; standard immunodepletion techniques were used. Normal human serum and Protein A Sepharose beads were used as controls.

Discussion

These studies demonstrate, by using immunological and functional criteria, that *Xenopus* oocytes have a Ku-like protein that is associated with the activity of DNA-dependent protein kinase (DNA-PK). In sea urchins, we have observed a similar association of immunologically identical protein subunits (unpubl. data). Although Ku homologs from *Drosophila* (Jacoby and Wensink, 1996) and yeast (Feldmann and Winnacker, 1993) have been described, their association with DNA-PK activity has not been clearly documented. Nonetheless, it appears likely that this holoenzyme has been conserved through evolution. Cross-reactivity between a Ku-like protein in *Xenopus* oocyte extracts and the monoclonal antibody 162, which recognizes the conformational epitope (p80/p70) of the human Ku, supports this notion.

Our results demonstrate that the oocytes synthesize more Ku-like protein during the late vitellogenic phase. This suggests that Ku may be one of the maternal proteins that must be stockpiled in abundance for subsequent use in embryogenesis. In full-grown oocytes (Y6), therefore, the radiolabeling of the immunoprecipitated Ku protein is not as prominent as in Y4 and Y5 oocytes. This may be attributed to a slowing or cessation of synthesis of Ku in Y6 oocytes that have already stored the previously synthesized Ku. The low level of DNA-PK activity detected in the pre- and early vitellogenic stages of oocytes is consistent with the finding of minimal levels of the

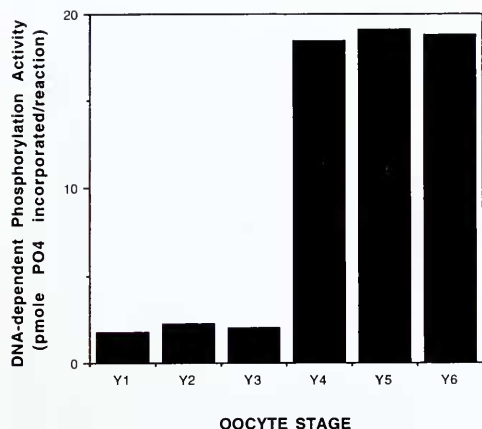

Figure 3. DNA-dependent protein kinase activity in oocytes at different stages. The isolated oocytes were homogenized as described in Materials and Methods (Immunoprecipitation and protein analysis). The total protein content of oocytes at each stage was equalized (140 μg) in all the extracts used for DNA-PK activity assay. The specific peptide was used as the substrate.

Ku protein detectable in these stages. Induction, using dsDNA, of the kinase activity in the oocyte extracts distinguishes in *Xenopus* an enzyme (DNA-PK) that is functionally similar to the one reported in human cell lines (Carter *et al.*, 1990). The association of this activity with a Ku-like protein is confirmed with immunodepletion techniques when anti-Ku antibodies remove the activity from the oocyte extracts. Ku protein and DNA-PK catalytic subunit (p460) remain as a complex at physiologic salt concentrations, but dissociate at high salt (0.5 *M*) concentrations (Gottlieb and Jackson, 1993). Therefore, we used anti-Ku antibodies coupled to Protein A-Sepharose beads to immunodeplete the holoenzyme from oocyte extracts prepared in low salt buffers. Extracts incubated with these beads lost the DNA-PK activity, whereas other nonspecific antibodies did not affect the activity. These results indicate that the Ku protein is associated with the DNA-PK activity observed in the oocyte extracts.

Although considerable evidence indicates that DNA-PK plays a role in DNA repair and recombination (reviewed by Anderson, 1993), the *in vivo* targets of the DNA-PK remain largely unknown. Additional studies in clarifying the behavior of the enzyme during early embryogenesis may be useful in recognizing specific targets of this enzyme. We are investigating whether the post-fertilization nuclear translocation of the enzyme activity reported in the sea urchin (Kanungo *et al.*, 1996b) also takes place in *Xenopus*; the results should elucidate the enzyme's specific cellular functions during development.

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